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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Aging is the single largest risk factor for developing breast cancer and is thought to be due the convergence of the accumulation of mutations together with the accumulation of senescent cells. Our working hypothesis is that senescent epithelial cells can cause preneoplastic or neoplastic changes in its neighbors, and that these changes will be manifest when cells are cultured in three dimensions, which more closely mimics the natural tissue environment than conventional two dimensional cultures. To test this hypothesis, we have successfully established two and three dimensional culture models of normal human mammary epithelial cells (HMECs) with and without a functional 16-tumor suppressor pathway. We have also created preneoplastic HMECs by introducing defined genes with oncogenic potential, particularly genes that selectively inactivate the p53 or pRB tumor suppressor pathways. We have used these, and frankly neoplastic human mammary epithelial cells, in the two and three dimensional co-culture assays in which presenescent HMECs are mixed with senescent HMECs and stromal breast fibroblasts.					
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## Table of Contents

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>9</b>
<b>Reportable Outcomes.....</b>	<b>9</b>
<b>Conclusions.....</b>	<b>9</b>
<b>References.....</b>	<b>9</b>
<b>Appendices.....</b>	<b>9</b>

## INTRODUCTION

Aging is the single largest risk factor for developing breast cancer. We have proposed that this risk is due to the synergy between the accumulation of mutations and the accumulation of senescent cells, which express an altered phenotype. An important feature of the senescent phenotype is the secretion of biologically active molecules that can disrupt the local tissue structure and function. In particular, we propose that senescent mammary cells can influence its neighbors, conferring on them preneoplastic or neoplastic phenotypes. Further, premalignant cells – that is, cells that have acquired one or more potentially neoplastic mutations – will be especially sensitive to the disruptive effect of neighboring senescent cells. We also hypothesize that changes caused by the presence of senescent cells will be most clearly manifest when the epithelial cells are cultured under conditions more closely mimicking the natural tissue environment than conventional cell culture systems. To test these ideas, we proposed to establish two and three dimensional culture and co-culture models of normal human mammary epithelial cells (HMECs) with and without stromal fibroblasts. We proposed to use HMECs with or without a functional p16/pRB tumor suppressor pathway, which is frequently spontaneously lost in cultured HMECs due to DNA methylation of the p16 promoter. We also proposed to create and incorporate into these culture models preneoplastic HMECs that lack functional p53 and/or p16/pRB tumor suppressor pathways owing to specific genetic modifications we introduce into the cells, and frankly preneoplastic and neoplastic human mammary epithelial cells. We proposed to monitor the cell culture models for indices of cell proliferation and differentiation, particularly morphological differentiation (ability to form structures present in normal or neoplastic breast tissue). We also proposed to monitor the phenotype of presenescent and senescent cells with or without functional p16 for markers of the senescent phenotype, including secreted factors that may influence the tissue microenvironment.

## BODY

1. In year 1, as reported in our progress report, we established and characterized for proliferation two dimensional cell culture models of presenescent and senescent normal human mammary epithelial cells (HMECs) that retain or lack a functional p16 tumor suppressor pathway and optimized culture conditions to obtain and manipulate cultures that were either presenescent and senescent (part of approved statement of work #1). We have subsequently explored ways to separate p16-dependent senescence from p53- and telomere-dependent senescence (also termed agonescence), and explored culture conditions to optimize their differentiation and ability to participate in, and/or influence neighboring cells, in three-dimensional alveolar morphogenesis (part of approved statement of work #2 and #3).

- a) We have explored a mechanism of cleanly separating senescence pathways in HMECs mediated by the p53 vs the p16/pRB pathway.

Research and findings: HMECs senescence owing to either of two mechanisms: 1) p16 induction by mechanisms that remain poorly understood, which engages pRB, or 2) telomere erosion and eventual dysfunction in cells that spontaneously silence p16 due to promoter methylation, which engages p53.

We have used primarily HMECs strain 184, but have also used cultures from other donors, specifically donor 48 or mixed donors.

We have compared cells cultured to replicative senescence, which required continual passaging for several weeks until there was no increase in cell number over a 2-week interval. At this point, cellular DNA synthesis declines to <10% over a three day interval (labeling index) and >75% of the cells express the senescence-associated beta-galactosidase (SA-Bgal), criteria for replicative senescence established in the previous funding period. We also used X-irradiation (5 Gy) to induce rapid senescence of HMECs, as described in the previous progress report. Both p16-dependent and telomere-dependent senescence results in similar loss of proliferation, DNA synthesis and SA-B-gal expression.

To determine the effect of purely p16-dependent vs purely telomere-dependent senescence, we used a dominant negative telomere-associated protein. TIN2 is a telomere-associated protein that interacts with the TRF1 telomeric DNA binding protein and is essential for telomere dysfunction. A truncated form of TIN2, termed TIN2-15C disrupts telomere function and induces immediate telomere-dependent senescence. A diagram of TIN2 and TIN2-15C is shown below, with the N-terminal, TRF1-interaction and C-terminal domains indicated.

We used TIN2-15C to induce rapid senescence in HMECs and fibroblasts. Since replicative senescence is thought to be due to both telomere dysfunction and subsequent engagement of a p53-dependent damage response and induction of the p16/pRB pathway due to stresses of unknown nature, the TIN2 mutant allowed us to induce senescence solely and synchronously by telomere dysfunction. This manipulation allows us to more cleanly separate the senescence-inducing pathways.

We created lentiviral vectors for efficient delivery of control and mutant TIN2 proteins. These vectors showed >90% expression in mammary epithelial and fibroblastic cells. In at least three experiments, we further demonstrated that TIN2-15C induced a telomere-dependent senescent response, as judged by <10% DNA synthesis and approximately 80% senescence-associated beta-galactosidase (SA-Bgal) expression. The micrographs below show cells infected with a control (left micrograph) and TIN2-15C (right micrograph) vector and stained for SA-Bgal, which is detectable as a blue color.

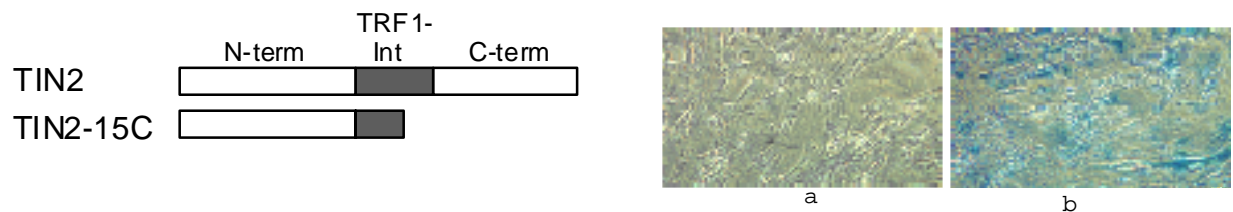


Fig 1

- b) We have defined and utilized culture conditions for HMECs, either preselected (p16+, p16 tumor suppressor pathway intact) or postselected (p16-, p16 pathway inactivated due to spontaneous methylation of the p16 promoter) HMECs, and either presenescent or senescent, using basement membrane components for induction of morphological differentiation in three dimensional cultures, as proposed, and determined the ability of senescent HMECs to participate directly in alveolar differentiation (morphogenesis) in three dimensions.

**Research and findings:** We co-cultured presenescent and senescent HMECs in three dimensional alveolar morphogenesis assays, using basement membrane components and GFP-expressing cells (described in the previous progress report) induced to senesce by replicative exhaustion, X-irradiation or synchronous telomere dysfunction owing to expression of TIN2-15.

In multiple attempts and over several experiments, we have shown that senescent HMECs do not participate in alveolar formation per se. Greater than 95% of the alveoli formed in these three dimensional cultures were composed of presenescent HMECs, with <5% of the structures containing GFP-marked senescent cells. This is an obvious negative finding. Nonetheless, it provides an answer to one of the hypotheses set forth in our proposal – namely, do senescent HMECs disrupt normal mammary epithelial cell morphogenesis as integral parts of alveola. The answer from our experiments is no. That is, the senescence response precludes active participation in alveolar morphogenesis, and the alveolar structures are rarely if at all affected by cell-cell contacts between presenescent and senescent mammary epithelial cells.

On the other hand, our negative result suggests that any effects of senescent HMECs in alveolar differentiation, then, are likely to be mediated by the diffusible factors produced by the senescent cells. This suggestion strengthens the utility of the antibody array methodologies we are developing (described in the previous progress report) to identify the specific factors secreted by cells induced to senescence by various inducers.

- c) We are continuing to characterize the effect of senescent fibroblasts in two and three-dimensional co-culture with p16<sup>+</sup> and p16<sup>-</sup> HMECs on the differentiation characteristics of the HMECs. Our results suggest that p16<sup>-</sup> HMECs can lose proper subcellular localization of the cell adhesion protein E-cadherin in the presence of senescent but not presenescent stromal fibroblasts. These effects were most apparent when the cells were co-cultured in three, as opposed to two, dimensions. In either case, however, in the presence of senescent fibroblasts, the HMEC maintained normal expression levels and distribution of the integral membrane protein alpha-6-integrin, indicating that the epithelial cells do not lose all differentiation markers. These results suggest that a senescent stromal microenvironment may promote loss of optimal epithelial cell-cell communication in the aged breast. Since the stromal cells were not in direct contact with the epithelial cells during the co-culture, these findings further implicate secreted factors as the important mediators of the morphogenic disruption caused by a senescent stroma.

Research and findings: We immunostained co-cultures of HMECs on presenescent and senescent fibroblast lawns with an anti-E-cadherin antibody, followed by staining with a fluorescent secondary antibody. E-cadherin immunostaining is generally most prominent at sites of cell-cell junctions. Likewise, we stained with an anti-alpha-6-integrin antibody, followed by staining with fluorescent secondary antibody. In the co-cultures, E-cadherin staining was less intense at cell-cell contact regions when postselected HMECs were cultured in the presence of senescent fibroblasts compared to culture in the absence of fibroblasts or presence of presenescent fibroblasts.

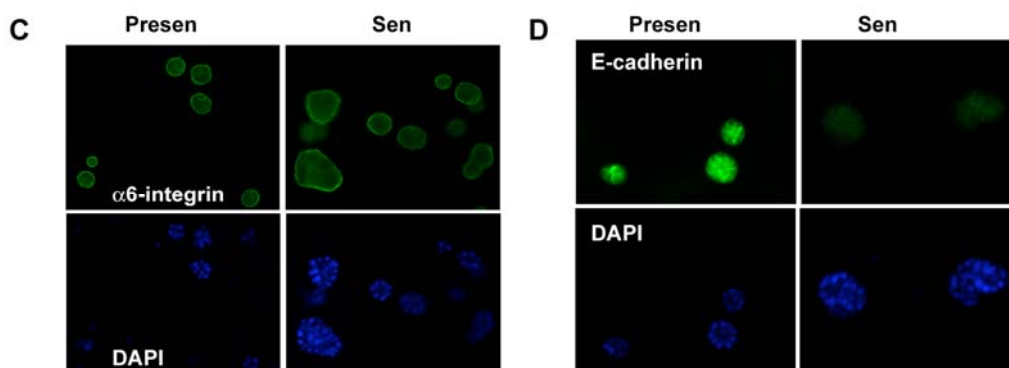


Fig 2

- d) We are continuing to optimize conditions to co-culture senescent and presenescent HMECs in two dimensions in order to determine the phenotypic consequences of the senescence of the epithelial cells on their epithelial neighbors.

Research and findings: In order to perform co-culture experiments with senescent epithelial cells, we must incubate them in medium containing no or minimal growth factors so that any growth stimulation we see can be attributed to the cells, not to exogenously supplied growth factors. We found that neither presenescent nor senescent postselected HMECs thrived in growth factor-free medium, as determined by the gradual appearance of a vacuolated cytoplasm and gradual loss of cell adherence to the culture dish. We have confirmed that supplementing the medium with low levels (10 ng/ml) of insulin help maintain viability, but this supplement is still sub-optimal. We have also tried supplementing with fetuin, which we found helped maintain the viability of mouse mammary epithelial cells, but fetuin has proven insufficient to fully sustain the viability of HMECs in serum-free medium. We plan to try other culture manipulations, including albumin and combinations of the above additives. So far, we have not optimized conditions to our satisfaction, but we plan to continue working on this problem until we are satisfied that we can maintain the cultures in a good enough state to obtain meaningful results.

2. Marker characterization of senescent cells using high-throughput assays of secreted factors expressed by senescent fibroblasts and HMECs (part of approved statement of work #1).

- a) As described in the previous progress report, we are optimizing the use of antibody arrays to characterize the factors secreted into conditioned medium from presenescent and senescent cells.

Research and findings: We are continuing to use the human arrays with which we initiated this study (commercially available and containing 120 antibodies directed against different human cytokines). We have completed our optimization, and are currently preparing a manuscript describing conditions for making the results quantitative. We have validated selected cytokines (e.g., interleukin-6, which is highly expressed by senescent cells) by enzyme linked immunoabsorbent assays, and have developed method to display and analyze the results by methods similar to those used for the display and analysis of cDNA microarrays. While the analysis is not yet completely finished, the results indicate that senescent mammary stromal cells secrete especially high levels of inflammatory cytokines such as interleukins-6 and 8, as well as certain matrix metalloproteinases (MMPs), particularly MMP3. Our next challenge is to apply this technology specifically to presenescent and senescent HMECs. This application will also require maintaining HMECs in a healthy state in serum- and growth factor-free media, since additives to the media would confound detection of factors secreted by the cells.

Shown below is a display of a part of one composite antibody array analysis in which we determined the relative secretion level of multiple factors by several different human fibroblast strains, including human mammary fibroblasts.

The signals from each antibody were quantified using a phosphorimager. The signals from the entire array were then averaged, and the quantification of signals above and below the average displayed colorimetrically. The cytokines, growth factors and other biologically active molecules that were overexpressed relative to the average are displayed as yellow. The factors that were underexpressed relative to the average are displayed as blue. Factors that did not change relative to the average appear as black.

The factors interrogated by the array are listed in the pale and dark gray boxes along the top of the display. The cells from which conditioned media were analyzed are listed in boxes to the left of the display. The cells included fibroblasts from fetal lung (WI-38, IMR90), neonatal foreskin (HCA2, BJ) and adult human mammary tissue (hBF). The human breast fibroblasts are indicated by the red arrows. The cells were either made senescent by X-irradiation (SEN) or were presenescent (PRE) but quiescent owing to incubation in serum-free medium. In addition, the cultures were maintained under either standard conditions of ambient (approximately 20%) oxygen, or in physiological (approximately 3%) oxygen.

The visual display allows easy processing of the many individual data points, and leads to several interesting conclusions. First, it is clear that many factors are secreted at higher levels by senescent cells relative to presenescent cells (more yellow in the senescent samples, more blue and black in the presenescent samples). There are, however, exceptions, including factors that are secreted at a lower level by senescent compared to presenescent cells. Second, there are somewhat more overexpressed secreted factors when cells are cultured in 20% compared to 3% oxygen. Since 20% oxygen is not physiological, despite its widespread use, and since oxidative stress can induce a senescence response, we conclude that standard culture conditions (20% oxygen) predisposes cells to express a partial senescence secretory phenotype. These results impel us to conduct future experiments using 3% oxygen culture conditions. Third, each fibroblast strain has its own secretory phenotype, whether presenescent or senescent. This is perhaps not surprising, given the varying tissues and donor ages from which the cells were derived.

As seen in the array analysis, under physiological oxygen, human mammary fibroblasts overexpressed a variety of the growth factors and cytokines interrogated by this array, including GRO1, interleukins 8, 7, 13 and 16, as well as factors such as IGFBP2 (insulin-like growth factor binding protein 2), among others.

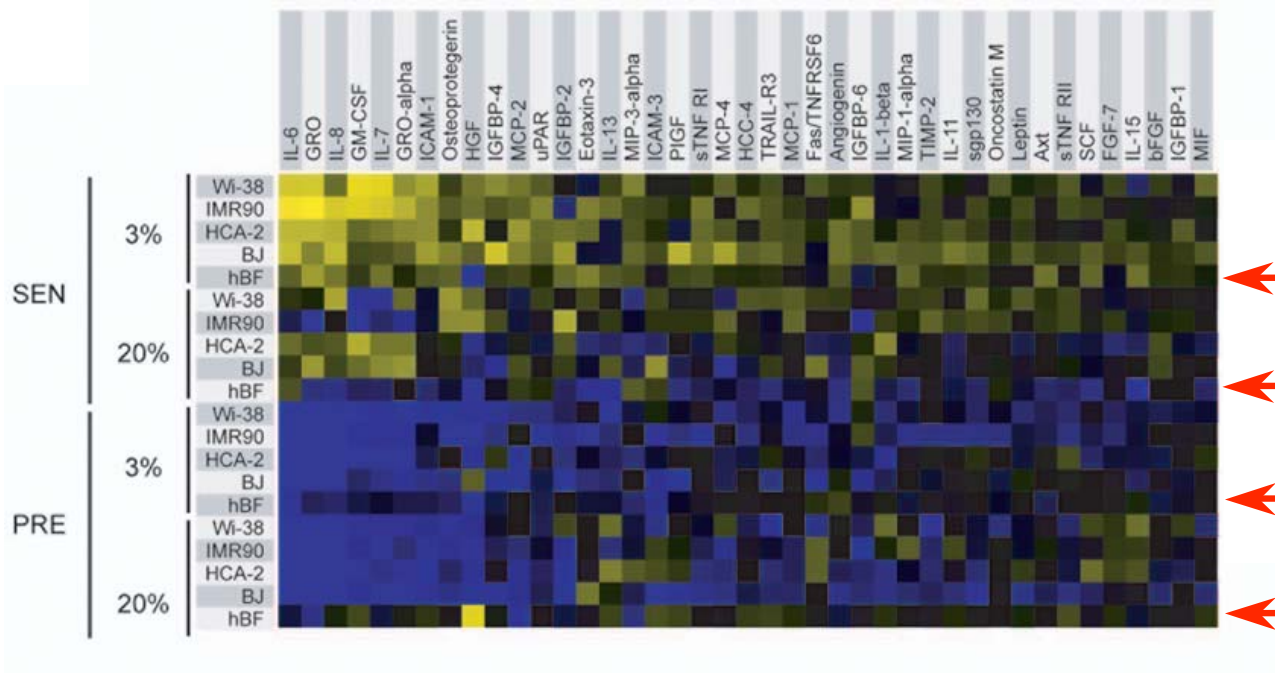


Fig 3

We have validated the array analyses by both immunostaining, where it is apparent that intracellular concentrations of the cytokines increase similarly to secreted concentrations, and



enzyme-linked immunoabsorbent assays (ELISAs), using selected cytokines. Results for two of the selected cytokines, interleukin 6 and 8 (IL6, IL8) are shown below for presenescent cells (PRE) and cells induced to senescence by X-irradiation (XRA) or replicative exhaustion (REP).

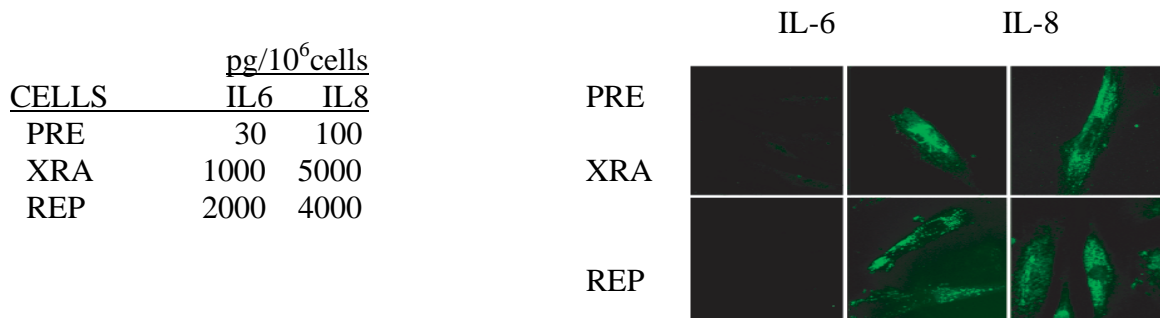


Fig 4

The ability to detect senescence-associated secreted molecules intracellularly by immunostaining suggests that at least some of these cytokines can be used as markers for the senescent state, both in culture and possibly in vivo.

### KEY RESEARCH ACCOMPLISHMENTS

- Determined that HMECs arrest with a senescent phenotype when synchronous telomere dysfunction is induced
- Determined that HMECs do not actively participate in alveolar morphogenesis in three dimensional culture
- Determined that senescent mammary fibroblasts can disrupt some but not all markers of differentiation in HMECs
- Optimized methods for quantification of antibody arrays to measure increased secretion of inflammatory and cell migratory cytokines by senescent cells.

### REPORTABLE OUTCOMES

No reportable outcomes thus far.

### CONCLUSIONS

We continue to make progress in establishing the proposed two dimensional and three dimensional culture systems of p16+ and p16- HMECs with or without stromal fibroblasts, showed that senescent HMECs do not directly participate in alveolar morphogenesis, and continue to characterize senescence markers in order to obtain a more comprehensive understanding of how senescent cells may alter HMEC phenotype and differentiation.

### REFERENCES

None

### APPENDICES

None